THE OXIDATION OF CYSTINE AND SOME RELATED SULFUR COMPOUNDS IN VITRO

By S. ALLAN LOUGH

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the University of Michigan

Reprinted from
THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 104, No. 3, Pages 601–610, 1934

THE OXIDATION OF CYSTINE AND SOME RELATED SULFUR COMPOUNDS IN VITRO

By S. ALLAN LOUGH

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the University of Michigan

Reprinted from
THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 104, No. 3, Pages 601–610, 1934

R. UNIV.
BIBLIOTHEEK
LEIDEN

Reprinted from The Journal of Biological Chemistry
Vol. 104, No. 3, March, 1934

THE REACTION OF NITROUS ACID WITH CYSTINE AND RELATED SULFUR-CONTAINING COMPOUNDS

By S. ALLAN LOUGH* and HOWARD B. LEWIS

(From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor)

(Received for publication, January 2, 1934)

Van Slyke in 1911 (1) observed that the amount of nitrogen obtained from the reaction of cystine with nitrous acid in the amino nitrogen determination was from 7 to 8 per cent greater than the theoretical amount. Schmidt (2), in a critical study of the reaction between nitrous acid and amino acids, observed that when a solution of cystine was mixed with nitrous acid and barium chloride a copious precipitate of barium sulfate appeared within a few minutes, and suggested that, as the oxidation of the sulfur of the cystine molecule took place, the nitrous acid was reduced with the formation of nitrogen as one of the products. The chief evidence in support of the hypothesis advanced by Schmidt (2) was afforded by the data obtained with β,β'-dithiodiglyceric acid (the desaminocystine of Neuberg and Ascher (3)). When this compound reacted with nitrous acid in the Van Slyke procedure, gas was evolved, and since this compound, if pure, contains no nitrogen, the implication was that any nitrogen thus obtained must have been derived from the reduction of nitrous acid. Two considerations, however, make this experiment less convincing than would have otherwise been the case; no figures are presented to indicate whether oxidation of the sulfur of β,β'-dithiodiglyceric acid to sulfate occurred and the purity of the acid (prepared by the deamination of cystine) was not satisfactory.

Sanchez (4) has suggested a series of reactions to explain the oxidation of the sulfur of cystine by nitrous acid in which the formation of pyruvic acid as an intermediate product of the reaction is postulated; but no adequate experimental basis for the re-

*On leave of absence from the University of Nevada, 1931–33.

actions proposed is presented in the publications available to us. Since Clarke and Inouye (5) have reported that pyruvic acid yields a gas in the Van Slyke apparatus, it seemed possible that the "extra nitrogen" might be related to the presence of the pyruvic acid formed as a secondary product of the reaction of the deamination of cystine by nitrous acid.

In the work to be reported in this paper, attempts have been made to approach the problem of the reaction between cystine and nitrous acid by securing data along three related lines. When cystine is acted upon by nitrous acid, what degree of oxidation of the sulfur can be obtained? By the examination of a series of organic compounds in which the type of sulfur linkage varies, is it possible to establish any relationship between the oxidation of the sulfur to sulfate by nitrous acid and production of extra nitrogen in the Van Slyke reaction? What is the chemical nature of the gas produced when nitrous acid reacts in the Van Slyke apparatus with a nitrogen-free sulfur-containing compound of known purity whose sulfur is readily oxidized to sulfate by nitrous acid?

EXPERIMENTAL

Determinations of amino nitrogen were made according to the micromethod of Van Slyke in the modified reaction chamber described by Koch (6) at room temperature (approximately 25°). The nitrous acid was allowed to react with the compounds studied for periods of 3, 30, and 60 minutes, with continuous agitation of the deamination chamber during the periods of 3 and 30 minutes. In the longer periods, the deamination chamber was shaken during the first 3 and last 3 minutes of the reaction period.

In the study of the oxidation of the sulfur of the various compounds to sulfate by nitrous acid, barium nitrite was used as the source of the nitrous acid. As the oxidation of the organic sulfur to sulfate occurred the barium ion thus introduced served to precipitate the sulfate ion. All the oxidations were carried out in beakers covered with watch-glasses in an incubator at $25° \pm 1°$. In order to maintain this temperature from the beginning of the reaction, all the solutions used were kept in the incubator before combining the materials in the reaction mixture. The transfer, ignition, and weighing of the barium sulfate formed were accomplished with the usual precautions.

The solutions of the sulfur compounds to be studied were prepared so that 20 cc. of the solution would contain approximately 50 mg. of sulfur, except in the case of a few compounds where insolubility or other factors made this impracticable. To 20 cc. of the solution of the substance under investigation, 20 cc. of water and 30 cc. of 3.25 per cent hydrochloric acid (0.975 gm.) were added and the mixture was carefully stirred. Barium nitrite (30 cc. of a 5 per cent solution, *i.e.* 1.62 gm. of crystalline barium nitrite) was added, the solution was carefully mixed, and placed in the incubator. The time of the addition of the barium nitrite was taken as the beginning of the period of oxidation. Every precaution was taken to insure as uniform conditions as possible for the series of experiments.

In the reaction mixture as described above, the excess nitrous acid decomposes presumably to form nitric acid, nitric oxide, and water, according to the equation, $3HNO_2 = HNO_3 + 2NO + H_2O$. The problem presented itself as to whether the nitric acid thus formed might not be responsible for the oxidation of the sulfur in the experiments. Control experiments in which barium nitrate replaced an equivalent amount of nitrite in the reaction mixture demonstrated that nitric acid, when formed in the reaction from nitrous acid, was not responsible for the oxidation of the sulfur of the cystine.

Since in the procedure outlined above the beakers were covered with watch-glasses only, the oxides of nitrogen formed escaped rapidly. It seemed probable that with the decrease in the amount of the reagent thus resulting, the rate of oxidation of the sulfur might also decrease. If this were the case, addition of more barium nitrite, after the reaction had progressed, should result in the oxidation of a larger proportion of the organic sulfur to sulfate sulfur at the end of a given period than in a reaction mixture similarly treated except that no extra barium nitrite was added. In such an experiment, in which cystine served as the source of organic sulfur, extra barium nitrite (15 cc. of a 5 per cent solution), acid, and water were added at the end of the first 5.5 hours of a 24 hour period of oxidation so that the concentrations of the barium ion and the acid were essentially constant. 49.5 per cent of the cystine sulfur was recovered as sulfate sulfur in the series which had received no additional barium nitrite; while in

the series to which extra barium nitrite had been added, 53.1 per cent of the sulfur was obtained as sulfate. In similar experiments the use of a higher concentration of nitrite (10 M sodium nitrite) did not increase the formation of sulfate sulfur from the oxidation of cystine.

In Table I are presented the data of two typical series of experiments in which the rate of oxidation of the sulfur of cystine to sulfate by nitrous acid was studied over short time intervals. It will be noted that the reaction proceeded most rapidly in the first 15 minutes during which 28 per cent of the total sulfur present

TABLE I

Oxidation of Cystine Sulfur to Sulfate Sulfur by Nitrous Acid

Duration of reaction	Series 1			Series 2		
	Total S of cystine	Weight of $BaSO_4$ obtained	Sulfate S of total S taken	Total S of cystine	Weight of $BaSO_4$ obtained	Sulfate S of total S taken
hrs.	*mg.*	*gm.*	*per cent*	*mg.*	*gm.*	*per cent*
0.25	51.32	0.1046	28.0	51.32	0.1061	28.5
		0.1049			0.1067	
0.50	51.32	0.1253	33.6	48.88	0.1247	35.1
		0.1259			0.1252	
1.50	51.32	0.1380	36.9	48.88	0.1380	38.7
		0.1379			0.1377	
3.00	51.32	0.1468	39.3	48.88	0.1426	40.1
		0.1468			0.1430	
6.00	48.88	0.1598	45.0	48.88	0.1550	43.6
		0.1602			0.1550	

was oxidized. No extra barium nitrite was added in these experiments.

Since the preliminary experiments had indicated that the extent of oxidation of the sulfur of cystine could be increased somewhat by the addition of more barium nitrite to the reaction mixture after some hours, an attempt was made to determine the maximum oxidation to sulfate sulfur which could be obtained by repeated addition of fresh nitrite. Two such series of experiments extending over 84 and 144 hours respectively were carried out in duplicate. In the first series, seven pairs of beakers containing the cystine, acid, and nitrite were prepared and incubated at

25° as outlined. After 12 hours, the contents of one pair of beakers were filtered off and the barium sulfate weighed. To each of the remaining beakers, 1.62 gm. of crystalline barium nitrite were added. After an additional 12 hour period, the contents of two more beakers were filtered off and the sulfate weighed. The remaining beakers received the same amount of barium nitrite as before. This procedure was continued until with the last pair of beakers 84 hours had been available for the oxidation and six extra portions of the nitrite had been added at 12 hour intervals. The acidity was regulated by the addition of hydrochloric acid (1:1) as required to keep the reaction definitely acid to litmus. The procedure of the second series which extended over a total of 144 hours or twelve 12 hour periods was similar. Despite the repeated addition of a fresh supply of the reagent at intervals, the rate of oxidation slowed up rapidly in each series and slight oxidation occurred after 72 hours. Under these conditions it was not possible to oxidize the sulfur of cystine completely to sulfate sulfur, a maximal oxidation of about 85 per cent of the sulfur being obtained in 144 hours.

The experimental procedures already described were applied to a study of the oxidation of a number of sulfur-containing compounds related to cystine. In those experiments in which the period of oxidation was of 24 hours duration, 1.62 gm. of crystalline barium nitrite were added to each beaker at the end of 12 hours. At the same time, the behavior of these compounds in the Van Slyke gasometric method for amino nitrogen was determined. The results are presented in Table II.

In the Van Slyke method, cystine yielded 15 to 20 per cent of nitrogen in excess of the amount present in the amino groups. These values are somewhat higher than those obtained by Van Slyke (1), but are probably to be explained in part by the longer periods of reaction and the higher temperature at which the reaction was carried out in our studies. Ready oxidation of the sulfur of the cystine occurred. When the carboxyl groups of cystine were esterified (*e.g.* cystine dimethyl and diethyl ester hydrochlorides), the reaction of the amino group with nitrous acid appeared to be delayed slightly in the short reaction period, although in the longer periods, amounts of amino nitrogen considerably in excess of those present were indicated. The sulfur of these ester hydrochlorides

was readily oxidized to sulfate by nitrous acid and to approximately the same extent as was the sulfur of cystine. When cystine had been oxidized to a sulfonic acid derivative, cysteic acid

TABLE II

Oxidation of Sulfur of Cystine and Related Sulfur Compounds by Nitrous Acid and Amount of Nitrogen Obtained from These Compounds by the Van Slyke Nitrous Acid Reaction for Amino Acids

In the case of compounds which contained no nitrogen, or which contained nitrogenous groups not reacting as primary amines with nitrous acid (*e.g.*, diacetylcystine), all the nitrogen obtained (Van Slyke) was "extra nitrogen." The percentages given were calculated by the expression, (gm.-atoms of N (Van Slyke)/gm.-atoms of S in sample) × 100.

Compound	Oxidation of S by nitrous acid Sulfate S of total S		N by Van Slyke procedure Amino N of total N		
	12 hrs.	24 hrs.	3 min.	30 min.	60 min.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Cystine	46.8*	57.5	114.9	117.7	120.0
" dimethyl ester hydrochloride	42.4	47.3	91.0	111.0	
Cystine diethyl ester hydrochloride					
Sample 1	39.7	46.7	94.8	116.5	
" 2	40.5	48.4	93.2	114.3	132.4
Cysteic acid		0.5	100.1	99.0	
Taurine		0.0	97.4	95.6	
S-Benzylcysteine	2.1	5.3	98.3	100.0	102.4
Diacetylcystine	4.1	7.0	1.6	1.9	1.5
Barium β,β'-dithiodiglycerate					
Synthetic, Sample 1†	6.4	10.7	0.0	0.0	0.0
" " 2‡	5.0	8.4	0.0	0.0	2.7
From cystine (5)	7.3	11.4	5.1	6.1	5.8
dl-Methionine		0.0	100.2	101.0	100.3
Thioglycolic acid	89.0	94.6	1.1	11.3	41.5§

* In 6 hours, 44.3 per cent of the sulfur was oxidized to sulfate.

† Prepared by Koelsch (7).

‡ Prepared in this laboratory by the method of Koelsch (7).

§ In a 2 hour period 55.0 per cent.

or taurine, neither formation of extra nitrogen nor oxidation of the sulfur by nitrous acid was to be observed (Table II). When the thiol group of cysteine was blocked by a benzyl group, as in S-

benzylcysteine, no extra nitrogen was obtained and the oxidation of the sulfur was so slight as to be almost negligible. Similar results were obtained with S-phenylcysteine. When methionine reacted with nitrous acid, no extra nitrogen and no oxidation to sulfate resulted. This was not unexpected in view of the findings with S-benzylcysteine. When the amino group was protected by an acetyl group (*e.g.* diacetylcystine), no significant amount of extra nitrogen and no oxidation of the sulfur to sulfate were observed.

The results obtained indicated a direct relation between extra nitrogen and oxidation of the sulfur to sulfate in the reaction with nitrous acid. A study of the behavior of compounds containing a thiol group but free from nitrogen afforded further evidence of this relationship. Thioglycolic acid (Table II) which contains no nitrogen gave more extra nitrogen than did any other compound studied and its sulfur was more completely oxidized, a conversion of 89 per cent to sulfate in 12 hours. Similar although slightly less striking results were obtained with α-thiolpropionic acid by Miss Wilma Marlowe in unpublished results from this laboratory. She also noted that in the reaction with thiodiglycolic acid, a compound which does not contain the thiol group and which does not yield this group readily on reduction, neither nitrogen nor sulfate was formed.

Schmidt (2) has reported that β,β'-dithiodiglyceric acid (desaminocystine) yielded extra nitrogen in the Van Slyke reaction, although in amounts much less than cystine itself. His compound, prepared from cystine by the action of nitrous acid, was not entirely pure. We have prepared the barium salt of this acid from cystine (3) and have studied its behavior, since the barium salt is more readily obtained in pure condition than the acid and since the presence of the barium was not objectionable in our procedure. The salt contained 35.95 per cent of barium (theory, 36.34 per cent) and 16.79 per cent of sulfur (theory, 16.99 per cent). When this compound reacted with nitrous acid, slightly less than 6 per cent of extra nitrogen was obtained. It will be noted, however, that contrary to the observations made with other sulfur compounds which reacted to give extra nitrogen, there was no significant increase in the amount of extra nitrogen as the reaction period was extended. Slight oxidation to sulfate was observed with the

formation of amounts of sulfate which we believe to be greater than the error of our experimental method. Due to the uncertainty as to the exact nature of the so called desaminocystine, we repeated our experiments with the barium salt of β,β'-dithiodiglyceric acid synthesized according to the method of Koelsch (7) using compounds prepared by Koelsch[1] and also in our own laboratory. Neither of these synthetic products yielded nitrogen in the reaction with nitrous acid. There was formed a small amount of sulfate, but the amount was clearly much less than the amount formed in the reaction between cystine or thioglycolic acid and nitrous acid. It is possible that the difference between the results obtained with the barium salt obtained from the deamination of cystine and the synthetic products may have been due to the presence of some substance other than the dithiodiglycerate in the deamination product. It seems possible that the α-keto derivative of cystine might be formed, in part at least, rather than the α-hydroxy derivative. Such a derivative, which differs from the α-hydroxy compound only in containing 4 less hydrogen atoms, would give essentially the same analytical values as the hydroxy compound. Since the sulfur of cystine is readily oxidized to sulfate by the action of nitrous acid, the failure of the sulfur of the β,β'-dithiodiglyceric acid to undergo a similar ready oxidation to sulfate suggests that at 25° this compound may not be the chief product of the reaction of cystine with nitrous acid. It is of interest to recall in this connection, that glycolic acid, the hydroxy derivative formed by the deamination of glycine, did not react with nitrous acid to form nitrogen (1), although glycine yielded an amount of nitrogen in excess of that present in the amino group.

Since the results had supported the hypothesis that extra nitrogen is obtained from sulfur compounds related to cystine only when oxidation of the sulfur to sulfate occurs, it seemed desirable to determine whether the gas was actually nitrogen or some other substance. It is obvious that a compound containing an amino group (*e.g.* cystine), which, when acted upon by nitrous acid should yield nitrogen, could not be used to establish the identity

[1] We wish to express our indebtedness to Dr. Koelsch and Dr. MacElvain of the University of Wisconsin for placing this material at our disposal. Because of the small amount available, we were unable to analyze this sample.

of the gas. It has been shown (Table II) that in the presence of nitrous acid, thioglycolic acid, a nitrogen-free compound, yields a considerable quantity of a gas which is not absorbed by alkaline permanganate. Thioglycolic acid was therefore chosen for this study. If the gas produced proved to be nitrogen and if this nitrogen appeared to be formed simultaneously with the oxidation of the sulfur of thioglycolic acid to sulfate, it would seem probable that the nitrogen originated as a product of the reduction of the oxidizing agent, nitrous acid. Moreover, in view of the similarity of the sulfur linkages in thioglycolic acid and cysteine (readily formed from cystine) and of the fact that sulfur of both thioglycolic acid and cystine may be oxidized readily to sulfate by nitrous acid, it would seem logical to conclude that the behavior of cystine is similar to that of thioglycolic acid with respect to extra nitrogen formation.

The reaction of thioglycolic acid and nitrous acid was carried out with all precautions to exclude nitrogen from sources other than the reaction, gases other than nitrogen were absorbed by the alkaline permanganate in the Hempel pipette, the residual gas was transferred to a Geissler tube and examined spectroscopically.

The band spectrum of the gas was photographed at 10,000 volts alternating current in the region from 8000 to 3300 Ångström units with a Hilger quartz spectrograph. All the bands were attributed to the nitrogen molecule. In spite of the fact that the plate was subjected to long exposure, there was no evidence of the presence of bands to be attributed to carbon monoxide, nitric oxide, or sulfur dioxide. It would appear, therefore, that of the gases given off during the action of nitrous acid on thioglycolic acid, the only gas not absorbed by alkaline potassium permanganate is nitrogen. This observation lends strong support to the belief that the "extra nitrogen" obtained from cystine in the Van Slyke reaction is nitrogen in reality.

An attempt is being made to measure simultaneously the extra nitrogen and the sulfate sulfur formed from the reaction of cystine and nitrous acid. If this attempt is successful, it should then be possible to depict more accurately the quantitative aspects of the reaction which forms the subject of this paper.

The spectrographic examination of the gas was made by Dr. R. W. Smith of the Department of Physics, to whom we wish to express our indebtedness.

SUMMARY

1. In an effort to explain the anomalous behavior of cystine in the Van Slyke gasometric method for amino nitrogen (1, 2), a study was made of the reaction of a series of sulfur-containing compounds with nitrous acid.

2. The sulfur of cystine was oxidized rapidly to sulfate by nitrous acid, but it was impossible to effect the oxidation of more than 85 per cent of the sulfur, even over a period of 144 hours and with the frequent addition of a fresh supply of the oxidizing agent.

3. Evidence was obtained to support the contention of Schmidt (2) that the extra nitrogen produced in the reaction between cystine and nitrous acid is related directly to the oxidation of the sulfur to sulfate with the reduction of nitrous acid to nitrogen.

4. When thioglycolic acid reacted with nitrous acid, the gas not absorbed by the alkaline permanganate in the Van Slyke determination was shown by spectroscopic examination to be nitrogen. Since thioglycolic acid resembles cystine in the Van Slyke reaction in that extra nitrogen is produced and the sulfur is rapidly oxidized to sulfate, it is believed that the "extra nitrogen" in the reaction between cystine and nitrous acid is in reality nitrogen, formed as a result of the reduction of the acid simultaneously with the oxidation of the sulfur.

BIBLIOGRAPHY

1. Van Slyke, D. D., *J. Biol. Chem.*, **9**, 185 (1911).
2. Schmidt, C. L. A., *J. Biol. Chem.*, **82**, 587 (1929).
3. Neuberg, C., and Ascher, E., *Biochem. Z.*, **5**, 451 (1907).
4. Sanchez, J. A., *Semana méd.*, **2**, 31 (1930).
5. Clarke, H. T., and Inouye, J. M., *J. Biol. Chem.*, **89**, 399 (1930).
6. Koch, F. C., *J. Biol. Chem.*, **84**, 601 (1929).
7. Koelsch, C. F., *J. Am. Chem. Soc.*, **52**, 1105 (1930).

WAVERLY PRESS, INC
BALTIMORE, U. S. A.